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TARGETING INVASIVE PROPERTIES OF MELANOMA CELLS

Imanol Arozarena[1] and Claudia Wellbrock[2,3]

[1]Cancer Signalling Group, Navarrabiomed-FMS, Calle Irunlarrea 3, Hospital of Navarra, 31008 Pamplona, Spain

[2]Manchester Cancer Research Centre, Faculty of Biology, Medicine and Health, The University of Manchester, Michael Smith Building, Oxford Road, Manchester, M13 9PT, UK

[3]corresponding author:
Claudia Wellbrock, Manchester Cancer Research Centre
Faculty of Biology, Medicine and Health, The University of Manchester, Michael Smith Building, Oxford Road, Manchester, M13 9PT, UK

Email: claudia.wellbrock@manchester.ac.uk

Tel: 0044-161-2755189

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Abbreviations

EMT	Epithelial Mesenchymal Transition
ECM	Extracellular Matrix
HGF	Hepatocyte Growth Factor
MAPK	Mitogen activated protein kinase
MITF	Microphthalmia associated Transcription Factor
MMP	Matrix-Metalloprotease
RGP	Radial Growth Phase
TGFb	Transforming Growth Factor beta
VGP	Vertical Growth Phase
W-RAMP	Wnt-mediated receptor-actin-myosin polarity

Abstract

Melanoma is a skin cancer notorious for its metastatic potential. As an initial step of the metastatic cascade melanoma cells part from the primary tumour and invade the surrounding tissue, which is crucial for their dissemination and the formation of distant secondary tumours. Over the last two decades our understanding of both, general and melanoma specific mechanisms of invasion has significantly improved, but to date no efficient therapeutic strategy tackling the invasive properties of melanoma cells has reached the clinic. In this review we assess the major contributions towards understanding of the molecular biology of melanoma cell invasion with a focus on melanoma specific traits. These traits are based on the neural crest origin of melanoma cells and explain their intrinsic invasive nature. A particular emphasis is given to lineage specific signalling mediated by TGFβ, and non-canonical and canonical WNT signalling, but also to the role of PDE5A and Rho-GTPases in modulating modes of melanoma cell invasion. We discuss existing caveats in the current understanding of the metastatic properties of melanoma cells, as well as the relevance of the 'phenotype switch' model and 'co-operativity' between different phenotypes in heterogeneous tumours. At the centre of these phenotypes is the lineage commitment factor MITF, one of the most crucial regulators of the balance between de-differentiation (neural-crest specific gene expression) and differentiation (melanocyte specific gene expression) that defines invasive and non-invasive melanoma cell phenotypes. Finally, we provide insight into the current evidence linking resistance to targeted therapies to invasive properties of melanoma cells.

Introduction

Cutaneous melanoma accounts for only less than 5% of all common skin cancers, yet it causes the majority of skin cancer deaths [1]. One of the main reasons for the lethality of melanoma is its metastatic propensity, which is partly reflected in the aggressive invasion of melanoma cells into neighbouring tissue at a time when the primary tumour is still significantly small in size. The invasive behaviour of melanoma cells appears to be a remnant of their neural crest origin; a trait that distinguishes this cancer from the other non-melanoma epithelial derived skin cancers. Because of their invasive potential, melanoma cells have been extensively used to study general mechanisms of cancer cell invasion [2-5]. Moreover, the idea of targeting melanoma cell invasion as means of therapeutic intervention stimulated an era of intense research with the aim of discovering the main regulator(s) of melanoma invasiveness.

During the last years the identification and specific targeting of genetic drivers of melanoma cell proliferation and survival -such as BRAF and other activators of the MAP-kinase pathway- along with the recent development and successes of immunotherapy has taken away the attention from ‘invasiveness’ as a crucial target for melanoma therapy. However, no therapy is unflawed, and patients who relapse with acquired resistance to BRAF and MEK inhibitors often present with melanomas that display a much more aggressive and invasive phenotype [6, 7]. Furthermore, not every patient responds to immunotherapy and again the phenotypes linked to innate resistance contain signatures linked to the invasive phenotype [8]. Hence, because invasive properties play important roles at every step of melanoma development, and

because an invasive phenotype appears to be linked to therapy resistance, there might still be a place for targeting invasive properties in melanoma.

Melanoma cells, melanocytes and the neural crest

Cutaneous melanoma is a cancer of transformed epidermal melanocytes, pigment cells that originate from the neural crest [9]. During development the expression of the Microphthalmia transcription factor (MITF) commits neural crest cells to the melanocyte lineage and marks melanoblasts in the dorso-lateral neural crest migration pathway [10]. These melanoblasts are highly motile, migrate throughout the embryo and colonize the basal layer within the epidermis, where they eventually differentiate into mature melanocytes. Post-migratory melanocytes are attached to the extracellular matrix (ECM) of the basement membrane of the epidermis and they exist in a homeostatic relationship with epidermal keratinocytes. Nevertheless, melanocytes still display a motile behaviour, although this is very much controlled by the neighbouring keratinocytes to which they closely adhere via cadherins, connexins and other adhesion receptors [11, 12]. However this control is lost in melanoma cells, allowing the migratory programme to be fully reactivated.

Dermal invasion, extravascular migration and EMT

At the early stages of melanoma development, transformed melanocytes display uncontrolled proliferation within the epidermis (radial growth phase, RGP), giving rise to melanoma in situ [11]. While melanoma in situ is not invasive, RGP melanoma cells are highly susceptible to molecular changes

and microenvironment derived signals that can stimulate their invasive properties and induce the vascular growth phase (VGP). In this context, the interactions with keratinocytes play a major role, and as mentioned earlier loss of these interactions supports detachment and invasion [13]. Moreover, keratinocytes can contribute to dermal invasion of melanoma cells; they produce HGF, which can down-regulate E-cadherin [14], secrete matrix-metalloproteinase 9 (MMP-9), which helps breaking down the basement membrane [15] and they can activate Notch signalling which induces invasion by up-regulation of miR-222/221 [16].

Invasion into dermal tissue allows intravasation and dissemination through the vascular route. However, melanoma also displays lymphatic invasion and angiotropism, which can bee seen as extravascular migration [17]. Lymphatic invasion has been detected in approximately 16-47% of invasive melanomas [18], and the occurrence of angiotropism has been reported in up to 70% of cases and is suggested as an independent prognostic marker predicting risk for metastasis [19, 20]. However, due to the lack of precise markers that could be used in routine analyses, so far no larger study has been conducted to assess the incidence of angiotropism [18]. Angiotropic melanoma cells migrate in a pericyte-like manner (pericytic mimicry) along extracellular surfaces of the vasculature without intravasating. Intriguingly, such behaviour is also observed during early neural crest cell/melanoblast migration. Little is known about the molecular players involved in extravascular migration, but angiotropic melanoma cells display a gene signature including neural precursor markers and regulators of migration such as *CCL2*, *ICAM1*, *IL6*, *SERPINEB2* and *PDGFR* [21]. A similar gene signature is induced by TNFα,

which in fact can stimulate angiotropism in the context of UV induced inflammation [22]. This observation provides a logical and important link between inflammation and melanoma cell dissemination [20].

The different gene signature features of angiotropic melanoma cells also reveal a very important aspect of melanoma cell motility. As mentioned above, melanoma cells are not of epithelial origin but derived from highly motile neural-crest cells, which had undergone ‘Epithelial Mesenchymal Transition’ (EMT) while leaving the neural tube. As a consequence, and despite specific differentiation at their final destination, epidermal melanocytes still express several EMT markers such as vimentin, N-cadherin, ZEB2 and SLUG, a property which is thought to predispose them to metastasis once they transform [23, 24]. Thus, when melanoma cells become invasive, they do not undergo a ‘classical’ EMT comparable to what is seen in epithelial cancers; rather it appears that de-differentiation towards their neural crest origin is required for motility [25]. Importantly however, while the requirement for de-differentiation is supported by many observations, what really defines the ‘de-differentiated state’ is less clear, i.e. is the ‘neural crest’ (MITF negative) state required or is the ‘melanoblast’ (MITF positive) state sufficient? After all it is the melanoblast and not the neural crest cell that performs long distance migration in the embryo. No thorough comparable study has addressed this important question, but an answer would certainly help to identify the markers crucial for melanoma cell invasion without distraction by putative melanoma ‘stem cell’ markers.

Specific drivers of melanoma cell motility and invasion

General regulators of the actin cytoskeleton that play a role in cancer cell motility and invasion such as SRC and FAK are also relevant in melanoma and various studies have shown that inhibition of these kinases will reduce melanoma cell invasion [26-29]. However, while the broad-spectrum tyrosine kinase inhibitor dasatinib, which also inhibits SRC kinases is effective in melanoma cells in pre-clinical studies [30], a phase II trial revealed that dose limiting toxicity is a major obstacle [31], which dampened the enthusiasm for targeting such general regulators, and stimulated research into more specific features of melanoma cell motility.

The chase for melanoma specific regulators of migration and invasion led to the identification of various factors and signalling events that control melanocyte lineage commitment and migration in early development. Amongst them are canonical WNTs (e.g. WNT3), Transforming Growth Factor beta (TGFβ) or non-canonical WNTs (e.g. WNT5A). Importantly, these factors not only directly impact on invasive behaviour by regulating the actin cyto-skeleton, but they also initiate cellular signalling that ultimately controls the expression levels and the function of the lineage commitment factor MITF [32].

The complex role of MITF in melanoma cell invasion

MITF is thought to be one of the most crucial regulators of the balance between differentiation (melanocyte specific gene expression) and de-differentiation (neural-crest specific gene expression). However, MITF's role in invasion is by far not clear.

It is assumed that MITF while inducing differentiation is a suppressor of invasion, and this is based on three facts: Firstly, it is well established that gene expression profiling of melanoma cells confidently identifies a highly 'invasive phenotype' characterised by extremely low MITF expression and consequently a signature of melanocyte de-differentiation linked to markers of neural crest, EMT and stemness [33]. The idea is that the phenotype linked to this signature possesses properties of neural crest cells, which explains the increased motility that these cells display. Secondly, and in line with the above, factors that reduce MITF expression, e.g. WNT5A, TGFβ or hypoxia [34-36] also increase the invasive potential of melanoma cells [37-39]. Importantly, genes like *WNT5A*, *TGFbeta*, their related signatures and a hypoxia signature are all included in the 'invasive/MITF-low' signature [33]. Last but not least and thirdly, the cell line 501mel -a cell line abundantly used in the field of 'MITF-research'- expresses extremely high levels of MITF (due to a *MITF* gene amplification and presence of mutated beta-catenin) and is poorly invasive. Moreover, MITF depletion from 501mel cells leads to increased invasion [40-42] and see Fig. 1A**.** Similar results are found with the high-MITF expressing non-invasive melanoma cells lines WM3682 and WM3526 [43] or the high-MITF expressing mouse cell line B16 [34]. Likewise, increasing MITF expression in 'invasive/MITF-low' melanoma cells (WM1716, WM3314 or WM266-4) suppresses invasion [40, 43].

While all the above facts seem to settle the case for MITF being a suppressor of invasion, so far nobody has examined whether MITF is actually required for invasion in the 'invasive/MITF-low' cells. This could in fact be the case as not only neural crest cells are motile, but also melanoblasts, which do express

MITF [10]. Indeed, we found that depletion of MITF in low MITF-expressing highly invasive WM266-4 melanoma cells leads to a dramatic decrease in invasion (Fig. 1A). Thus, there might be a pro-invasive role for MITF after all. Indeed, MITF regulates the expression of a large set of genes that are linked to the GO terms ‘actin cytoskeleton’, ‘migration’ and ‘invasion’, and while in cell lines expressing high levels of MITF it suppresses the expression of these genes, in cell lines with low MITF expression levels, it actually induces them (Fig. 1B and unpublished data).

A pro-invasive role for MITF is also supported by the fact that the receptor c-MET that mediates Hepatocyte Growth Factor (HGF) stimulated melanoma cell invasion is a MITF target gene [44, 45], and that ectopic MITF overexpression increases 501mel cell invasion in response to HGF [45]. The ‘melanoma predisposition’ mutant $MITF^{E316K}$ also enhances 501mel invasion [46]. Finally, in our hands many cell lines considered to belong to the MITF expressing (MITF-high) group display invasive behaviour in 3D extracellular matrix (ECM) systems.

In summary, the role of MITF in invasion appears to be more complex than generally assumed, and ‘invasive’ signatures linked to low MITF expression as well as the fact that factors that induce invasion also down-regulate MITF are muddying the water. In an era of ‘omics’ profiling signatures are surely very helpful, but the case of MITF highlights that the relationship between ‘signature’ and functional behaviour’ might not always be as simple as assumed. It seems to fully comprehend MITF’s role in invasion its expression and function has to be seen in context of each particular signalling network.

The Yin and Yang of non-canonical and canonical Wnt signalling

One such signalling network is downstream of non-canonical WNT with WNT5A being a major player [47]. In the conjunction with ROR2, WNT5A binds to frizzled receptors and drives invasion through intracellular Ca2+ and protein kinase C (PKC) [38, 48] (Fig. 2). WNT5A can control directional movement by activating localised Ca2+-induced actin myosin contraction. This occurs through RHOB and the Wnt-mediated receptor-actin-myosin polarity (W-RAMP) structure, which contains actin, myosin IIB and melanoma cell adhesion molecule (MCAM) [49, 50]. Furthermore, WNT5A induced Ca2+ signalling can stimulate calpain1-mediated cleavage of the actin cross-linker filamin-A [51]. Thus, WNT5A has a major impact on actin cytoskeleton dynamics. Moreover, a role for WNT5A in vesicular trafficking in melanoma cells is also seen in the CDC42 dependent release of exosomes, which amongst other proteins also contain MMP2 [52], suggesting that WNT5A also contributes to the remodelling of the ECM.

In addition to its role in actin and ECM dynamics, WNT5A induces expression of vimentin and SNAIL and suppresses PAX3, a transcriptional regulator of MITF, and thereby reduces the expression of melanoma differentiation genes [35] (Fig. 2). The 'Yang' to non-canonical Wnt signalling in melanoma is canonical Wnt signalling (Fig. 2), which is required for early melanocyte lineage commitment and differentiation by inducing beta-catenin mediated expression of MITF [53]. Beta-catenin mutations in melanoma are rare, and nuclear beta-catenin expression has been linked with good prognosis [54, 55]. Moreover, mutated/stabilised beta-catenin induces high MITF expression, and MITF blocks the pro-invasive activity of beta-catenin [40]. In line with this,

overexpression of a stabilised beta-catenin mutant in the melanocyte lineage of mice in which melanoma development is either driven by NRas or $Braf^{V600E}$/Pten results in primary tumours with a low degree of invasiveness and high degree of pigmentation, an indicator of differentiation [56, 57]. Intriguingly, however the presence of stabilised beta-catenin in these mice dramatically increases metastatic burden and the observed metastatic tumours are highly pigmented [56, 57]. The exact mechanisms underlying this phenomenon are so far unknown, but it suggests that reduced ability to invade does not necessarily preclude metastatic potential. Possibly, high proliferative activity or prevention of anti-tumour immunity, which both have been linked to beta-catenin mutations in melanoma [58, 59] could also be a determinant for metastatic behaviour.

BRN2 controls PDE5A, a suppressor of melanoma cell invasion

An important suppressor of melanoma cell invasion is the cGMP-specific phosphodiesterase PDE5A, which removes the cGMP required for Ca2+ triggered actin-myosin contractility and invasion [60]. In line with such a suppressor role, a follow-up prospective cohort study linked the use of the PDE5 inhibitor sildenafil with an increased risk of melanoma, a correlation still under debate [61]. As PDE5A is a negative regulator of invasion, its transcriptional suppression, which is executed by BRN2 (Fig. 2), is correlated with increased invasion [60]. This reveals BRN2 as a positive regulator of invasion, which is in agreement with the observation that expression from the *BRN2* promoter is increased in motile cells, when analysed in vivo by intravital imaging [62]. This study also assessed a potential relationship between the differentiation state (i.e. pigmentation) and BRN2 expression in motile cells,

again with the idea that motile cells are more de-differentiated, and possibly BRN2 could contribute to this phenotype. Indeed, pigmentation was greatly down-regulated in motile cells, however this was not significantly correlated with the increase in activity from the BRN2 reporter [62], suggesting that reduced pigmentation and increased BRN2 expression are two independent events linked to invasion.

TGFβ and the 'invasive' phenotype

Pinner and co-workers also found that TGFβ signalling suppressed pigmentation and induced migration [62]. This observation is in line with the fact that 'active TGFβ signalling' is a key determinant of the 'original' invasive/MITF-low signature, described by Hoek and co-workers [33]. In agreement with a role in de-differentiation, TGFβ can maintain the melanocyte stem cell state by directly suppressing the expression of PAX3 (and hence MITF, see Fig. 2) in melanocytes [63]. TGFβ can also suppress MITF expression through GLI2 [64] or through CITED1 [65]. So far, these observations are all in agreement with a simple model in which TGFβ suppresses MITF expression and drives cells towards the de-differentiated and invasive phenotype. However, several findings suggest that this model is in fact not that simple and that possibly the de-differentiation (achieved through suppression of MITF) and the regulation of invasion are two independent activities downstream of TGFβ. For instance, we have shown that in melanoma cells TGFβ also suppresses PAX3 and consequently MITF [66], but we find that in many melanoma cell lines, despite efficiently down-regulating MITF TGFβ does not increase invasion (unpublished data).

Furthermore, while manipulating GLI2 impacts on melanoma cell invasion, whether the suppression of MITF downstream of TGFβ is actually required for invasion to occur has not been shown [64]. In addition, despite being able to suppress MITF expression, CITED1 displays significant co-expression with MITF [65], and while this predicts that only low CITED1 expression is linked to the 'invasive' signature, high expression of a CITED1 specific gene signature [65] as well as CITED1 itself [67] is correlated with poor prognosis. This might be due to the fact that apart from suppressing MITF, CITED1 is actively involved in TGFβ stimulated melanoma cell invasion by inducing the transcription of genes encoding important actin cytoskeleton regulators such as ARHGEF5 and MRIP, which regulate cortical actin myosin contraction [67]. Thus, while TGFβ induced signalling is clearly linked to invasion, whether the suppression of MITF and its target genes is required for its pro-invasive activities is so far unclear.

The role of RHO GTPases in melanoma invasion

Although there is broad evidence on the key role of RHO-ROCK signalling in melanoma cell invasion no activating mutations have been found in RHO in human melanoma. On the other hand, RAC activating mutations are present in 4-7% of human melanomas with the mutation P29S in RAC1 being the third most recurrent human melanoma mutation after $BRAF^{V600E}$ and $NRAS^{Q61L}$ [68, 69]. In vitro studies show that while the P29S mutation leads to RAC1 GTPase hyperactivation and lamellipodia formation, it actually reduces RAC´s ability to form invadopodia and modify the extracellular matrix [70], questioning that the P29S mutation contributes to melanoma development

predominantly by acting on invasion. Studies in transgenic melanoma models have demonstrated that although active RAC cannot drive melanoma, it cooperates with oncogenic RAS to promote melanoma proliferation and invasion [71, 72]. In line with these reports the RAC-specific exchange factor PREX-1 is over-expressed in human melanoma and drives melanoma metastasis while another member of the family, PREX-2 is found mutated in human melanoma, increasing its enzymatic activity towards RAC1 to drive gene expression and the cell cycle by activation of the PI3K pathway [73-75].

Because melanoma cells are extremely motile and invasive cells, it is not surprising that they have been widely used to study the cell intrinsic mechanisms underlying cancer cell invasion. By analysing melanoma cells under conditions resembling the 3D ECM two major contributions to cellular invasion have been identified (Fig. 3). As such, cells can undergo cell-shape changes in order to adjust to the 3D-architecture of the tumour microenvironment and they can modulate the ECM through protease activities, such as matrix-metalloproteinases (MMPs) [2-5]. Considering these contributions, cells can invade in a mode that is regulated through integrin-mediated adhesion and is limited by their MMP activity, or a mode that uses cell-shape changes and is less restricted by protease activities [2, 76]. The latter mode of invasion is represented by a round cell-shape that shows reduced substratum adhesion, and is associated with RHO dependent and phospho-MLC driven actin cortex contractions and formation of membrane blebs [3, 77]

What makes melanoma cells so efficient in invasion is the plasticity that is observed between the two modes of invasion described above (Fig. 3), as this

allows the cells to perfectly adapt to the extracellular environment [4]. The switch between modes of invasion is regulated by the interplay of RHO GTPase exchange factors and GTPase activating proteins, whereby RHO activation leads to RAC inactivation and vice versa to control melanoma cell invasion plasticity [78]. Interestingly, while elongated cells use integrins and are absolutely dependent on MMPs to invade, also contractile cells express and secrete MMPs (MMP-13, MMP-9) and their invasion has been shown to be regulated by both enzymatic and non-enzymatic protease signalling [79].

Differential signalling through RHO or RAC has also been linked to the overall mode of melanoma invasion, whereby the plasticity in RHO and RAC driven modes of invasion would support single cell invasion. Single cell invasion is considered a characteristic feature of melanoma cells as their non-epithelial nature suggests a low degree of cell-cell adhesion, allowing cells to invade 'freely' without contact to other cells. Single cell invasion can be observed in experimental settings in vitro and in vivo [27, 62, 78, 80]. Moreover, while it is difficult to draw conclusions from the non-dynamic situation found in human melanomas fixed for analysis, single cells can be seen even in histological sections of invasive melanoma (www.proteinatlas.org).

What is often observed in melanoma biopsies, are groups of cells that have invaded the dermis (see Fig. 3**)**, which could be the result of collective invasion. Collective invasion is typically detected in epithelial tumours, and occurs when cell-cell contacts are maintained and polarise migrating cells into a multicellular coordinated unit, which is driven by RAC mediated activities [81]. As mentioned above due to the non-epithelial nature of melanoma cells, instead of being the result of collective invasion, the observed invaded cell

clusters could also be the product of some individual cells that started to proliferate after an initial invasive phase. Nevertheless, collective invasion appears to occur when cells break through the epidermal basement membrane and start invading the dermis (see Fig. 3).

Phenotype switching and co-operativity

Regardless of whether single cell or collective activities are driving melanoma cell invasion, a question that is still left unanswered is whether melanoma cells have to change their phenotype in order to participate in invasion. Indeed, melanoma cells can undergo what is called ‘phenotype switching’, a process similar to ‘EMT’ that is observed with cells of epithelial origin [82]. In the concept of ‘phenotype switching’ MITF takes a central role as its abundant expression and the expression of many of its target genes (regulating cell cycle progression and pigmentation) is defining the so-called ‘proliferative phenotype’ [33]. The gene signature linked to the proliferative phenotype is void of genes that are linked to ‘invasion’ and ‘TGFβ’ and hence describes a non-invasive phenotype [33]. As mentioned earlier, the down-regulation of MITF is believed to initiate the ‘invasive phenotype’. In the ‘phenotype switching model’, cells switch between proliferative and invasive phenotypes throughout melanoma progression (Fig. 4), whereby the MITF-low/invasive phenotype performs invasion and dissemination, and cells switch back to the MITF-high/proliferative phenotype at the metastatic site in order to proliferate [82].

Intriguingly, using zebrafish transplantation assays we made the striking observation that under heterogeneous conditions melanoma cells differing in

their MITF expression levels (low and high) collaborated in their invasive behaviour, which allowed cells classified as non-invasive 'proliferative phenotype' to co-invade with cells of the 'invasive phenotype' [80]. This concept, which we termed 'co-operative invasion' (Fig. 4), has been observed in other settings where individual cancer cells cooperate to drive tumour progression [83-85] The cooperation between individual cancer cells in a primary tumour might explain the observed heterogeneity in secondary tumours with regard to the mitotic, invasive and metastatic competence of distinct cell populations. In the context of invasion, we found that the invasive cells provided the non-invasive cells with MMPs, thereby allowing the proliferative phenotype to prevail [80]. Performing such co-operative behaviour suggests that no further switch is required, and when cells of the proliferative phenotype arrive at the secondary site they can proliferate. Importantly, we observe that when cells cooperate during invasion, reciprocal interactions alter the overall invasive behaviour of a tumour, which suggests that the current definitions of melanoma cell lines as invasive and non-invasive/proliferative are limited in the context of heterogeneity.

As we are only able to observe 'stills' of melanoma progression when analysing histological sections, it is impossible to state whether a metastasis is the result of 'phenotype switching' or 'co-operativity'. However, the fact that circulating melanoma clusters consisting of cells with high and low MITF expression have been isolated from patients [86], and the enormous phenotype heterogeneity that is observed in metastatic melanoma supports the idea that co-operativity occurs throughout melanoma progression.

Invasion and therapeutic approaches

At present the only clinical option to prevent the appearance of metastatic disease if melanoma is diagnosed early, is surgical resection of the primary tumours and/or lymphatic nodes and adjuvant chemotherapy based on high dose interferon 2b, which has very little proven efficacy [87]. Despite the high metastatic potential of melanoma and the understanding of the molecular mechanisms governing melanoma cell invasion only a handful of clinical trials have attempted to target this step of the metastatic cascade. Indeed trials testing the multi-kinase inhibitor dasatinib failed due to excessive toxicity while the SRC specific inhibitor saracatinib provided no clinical responses [31, 88]. In the same line early phase trials assessing drugs targeting invasion through integrin signalling or MMP activity inhibition were not successful against advanced melanoma [89, 90]. RAC inhibitors have been assessed in pre-clinical studies but not yet in clinical trials [91]. On the other hand the central role played the RHO-ROCK signalling in invasion and metastasis has led to the development and characterization of ROCK inhibitors as a mean to block melanoma progression although it is yet to be defined whether the antitumor effect observed by ROCK inhibitors in mouse models of metastatic melanoma is uniquely due to its ability to inhibit contractile-dependent melanoma cell invasion or other biological processed regulated by ROCK such as intra- or extravasation, cell cycle progression and/or cell viability [79, 91-93].

Invasion and MAPK pathway targeting therapy

In the last few years the crosstalk between the molecular mechanisms governing melanoma cell invasion and resistance to targeted therapies

against component of the MAP-kinase (MAPK) pathway has gained growing attention. In melanoma the *BRAF* proto-oncogene is mutated in around 44% of patients and BRAF and MEK inhibitors have been shown to produce profound but transient clinical responses [94, 95]. In the majority of cases patients relapse due to reactivation of the MAPK pathway but also by the induction of compensatory pathways such as the PI3K/AKT cascade to sustain melanoma growth [96]. Furthermore, as a direct consequence of BRAF inhibition, increased activity of ROCK1 is observed, which appears to be due to reduced expression of RND3 when the MAPK pathway is inhibited [97, 98]. Despite increased ROH/ROCK activity inhibitor treated cells display an elongated shape and display increased invasion, a phenomenon that is also observed when melanoma cells are treated with MEK inhibitors, where the increased invasion is dependent on integrins and MMPs [27, 99]. Moreover paradoxical activation of the MAPK pathway by BRAF inhibitors in NRAS mutant melanoma cells leads to increased invasion and metastatic potential, while BRAF mutant melanoma cells selected for their resistance to the BRAF inhibitor vemurafenib show increased invasion through reactivation of the MAPK pathway, again in a protease (and hence integrin) dependent mode of invasion [100]. It is therefore not surprising that the context of the increased invasion observed upon MAPK pathway, SRC kinase activation seems to play a key role. This observation has led to the proposal of combinatorial therapies based on both MAPK inhibitors and SRC inhibitors to tackle both invasion and growth [27, 99, 101]. Following this scientific rationale broad-spectrum panRAF inhibitors that also show activity against SRC are being tested [102]. Interestingly, there might even be a crosstalk

between actin cytoskeleton regulators and MAPK signalling with regard to melanoma cell survival as combination of ROCK inhibitors with either BRAF or MEK inhibitors enhances cell killing [98, 103].

Among tumours progressing on MAPK inhibitors, approximately 50% show up-regulation of receptor tyrosine kinases such as AXL or EGFR, and concomitant reduced expression of the transcription factor MITF [7, 104, 105], which leads to resistant tumours with a de-differentiated, invasive phenotype [7]. Enormous effort is put into understanding the drivers of this particular phenotype, and the idea of targeting receptor tyrosine kinase signalling is currently considered as therapeutic option. However, there is a clear need to better understand the signalling that is downstream of these receptors and that is linked to the 'invasive' signature found in these resistant tumours.

Concluding remarks

Melanomas are highly metastatic skin tumours and metastatic disease is notoriously difficult to manage. As one of the first steps in the metastatic cascade, invasion has been the focus of intense research over the past 20 years, and melanoma has often been used as a model for cancer cells with invasive capacities. The molecular mechanisms governing melanoma cell invasion have unveiled how plastic these cells are depending on changes in the microenvironment, and the neural crest origin of melanocytes seems to be at the bottom of the high capacity of melanoma cells to disseminate. While we have gained a good understanding of the molecular mechanisms by which melanoma cells invade, there are still important questions that remain unanswered.

It is generally believed that tackling invasion can prevent the development of lethal metastases, but a growing number of reports using pre-clinical models or describing the results of detailed gene expression analyses suggest a weak correlation between the phenotype of invasive cells and that of cells from metastatic sites. Mechanisms such as phenotype switching or co-operativity as metastatic strategy could explain these discrepancies, and therefore targeting these processes might be more appropriate, and we need to invest efforts into dissecting these mechanisms and identifying the key players.

What should not be forgotten is that from a clinical point of view the suitability of “invasion” as the target for anti-metastatic oriented therapies, particularly for melanoma, is debateable. First, the plasticity of melanoma cells that enables them to adapt to new environments and to switch modes of invasion upon therapeutic intervention poses an intrinsic difficulty to stop invasion. Second, from a purely strategic point of view it has not yet been determined how important invading capacities are for the overall metastatic potential of melanoma cells. This is reflected for instance in the fact that beta-catenin mutations result in less invasion, yet a greater metastatic potential. Indeed, the metastatic cascade is a complex, multistep process that requires many other biological skills (such as adhesion to blood vessels, resistance to anoikis in the blood flow, proliferating activities). However, currently a major limitation to fully understand the relevance of invasive activities for the metastatic potential of melanoma cells is the fact that the majority of cell lines used in the melanoma community to study these processes are derived from lymph node or skin metastases, sites that do not necessarily reflect the lethal metastases of melanoma. Indeed, a thorough comparative study between

primary tumours and distant metastatic lesions is lacking due the scarcity of samples obtained from distant sites such as the brain, liver or lung. Even in the over 300 samples utilised in the TCGA study [106] only nine corresponded to distant organ metastases, the majority are from lymph node and skin metastases. Undertakings such as the 100,000 Genomes Project (www.genomicsengland.co.uk) aim to close this gap, which is urgently needed in order to enable us to target the properties that make melanoma cells so metastatic. Clearly, genes included in the 'invasive' signature are contributing to the 'aggressiveness' of melanoma as this situation is found in tumours resistant to current therapies, so identifying these players might also help to improve the current standard of care.

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Author contributions

Both authors discussed and developed the concept of the review and wrote the manuscript.

Conflicts of Interest

None

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Figure Legends

Figure 1. The effects of reducing MITF expression levels on melanoma cell invasion. A. RNAi mediated reduction of MITF expression increases invasion of MITF-high 501mel cells and reduces invasion of MITF-low WM266-4 cells into 3D dermal collagen. Both cell lines express GFP and have been analysed using FluoroBlok inserts coated with collagen gels. B. Model indicating the opposite function of MITF in MITF-high and MITF-low cells.

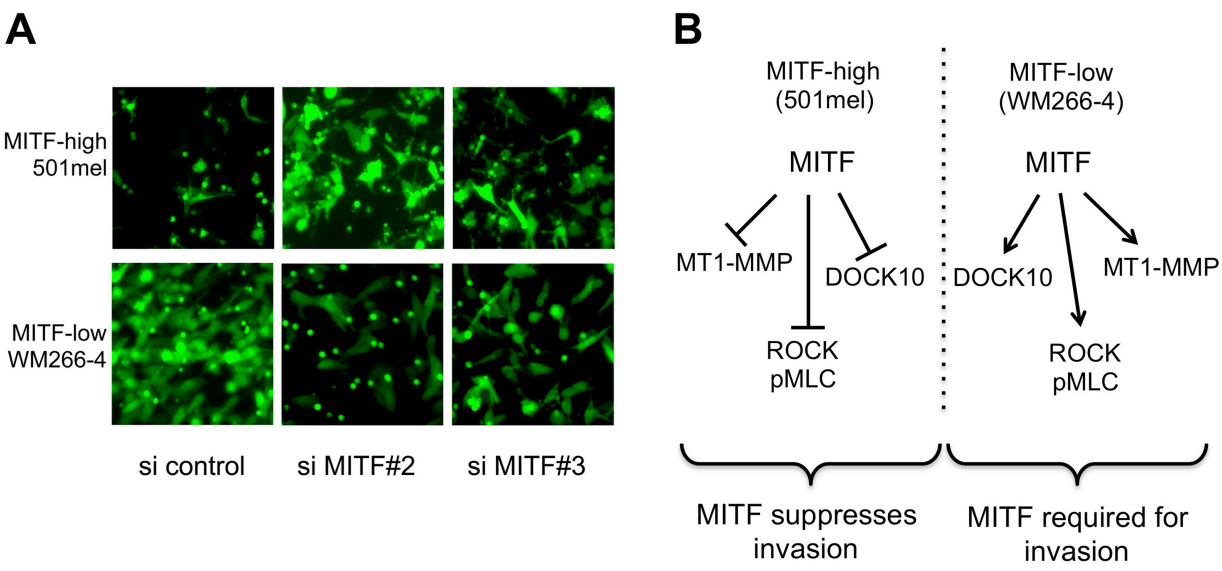
Figure 2. Signalling that activates melanoma cell motility and invasion. TGFβ stimulates the activation of SMAD2, which together with the co-factor CITED1 induces the expression of genes that regulate RHO/ROCK mediated contractility and invasion [67]. WNT5A can directly regulate the actin cytoskeleton and hence motility through calpain-mediated cleavage of filamin A (FLNA) [51], and regulate invasion through the induction of various genes downstream of PKC [48]. WNT5A also inhibits canonical WNT3A signalling, which otherwise suppresses invasion partly by inhibiting MT-MMP expression [40]. WNT5A also regulates vesicular trafficking and thus contributes to the release of MMP2 containing exosomes [52] as well as to the localisation of the W-RAMP structure to the edge of the cell [49, 50]. Downstream of TGFβ SMAD2 also suppresses PAX3, a transcriptional regulator of MITF [104], and CITED1 can also suppress MITF [65]. PAX3, and consequently MITF are also suppressed by WNT5A through the activation of STAT3 [35],

however BRN2 which is a positive regulator of invasion by suppressing the RHO inhibitor PDE5A [60] induces MITF expression [107].

Figure 3. Different modes of melanoma cell invasion. An illustration depicting various patterns of melanoma cell dermal invasion based on what can be seen in histological sections of melanoma lesions is shown. As these sections represent stills it is not clear whether the 'nests' of melanoma cells often seen in the dermis are the product of 'collective' invasion or of 'single cell' invasion followed by a proliferative phase. Clearly 'single cell' invasion can occur as such cells can be detected in melanoma specimens. Form experimental settings, we know that a more rounded shape of melanoma cells can be observed when cells possess high levels of RHO activity contracting cortical actin. In a dynamic process during invasion such cells can switch to a more elongated shape where high RHO activity is located at the rear of the cell, whereby invasion is driven by RAC activity at the front [4]. Elongated cells require MMP activity whilst invading by using integrins, whereas rounded cells can use membrane blebs for invasion, but this is aided by the presence of MMPs [79].

Figure 4. Phenotype switching and co-operativity. Phenotype switching is thought to follow altered MITF expression in order to generate different phenotypes with only one phenotype being compatible with a particular stage of tumour progression [82]. During co-operative invasion, an initial switch creates an invasive cell, which then can co-operate with cells that have not undergone a switch to enable them to invade as well [80].

Figure 1


A
MITF-high
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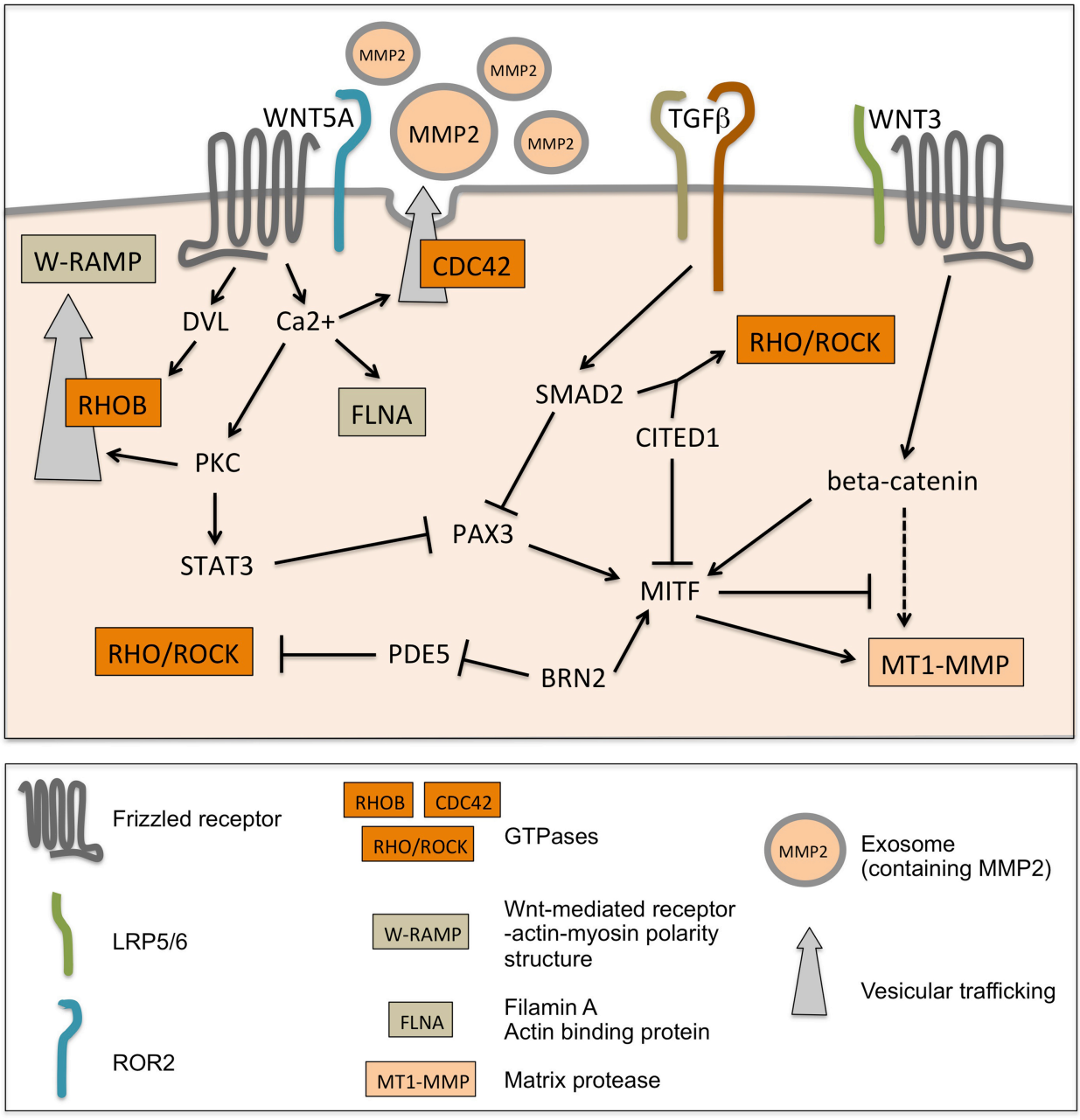
MITF-low
WM266-4
si control
si MITF#2
si MITF#3
B
MITF-high
(501mel)
MITF-low
(WM266-4)
MITF
MITF
MT1-MMP
DOCK10
ROCK
pMLC
DOCK10
MT1-MMP
ROCK
pMLC
MITF suppresses
invasion
MITF required for
invasion

Figure 2


MMP2
MMP2
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WNT5A
TGFβ
WNT3
W-RAMP
CDC42
DVL
Ca2+
RHO/ROCK
RHOB
FLNA
SMAD2
CITED1
PKC
beta-catenin
PAX3
STAT3
MITF
RHO/ROCK
PDE5
BRN2
MT1-MMP
Frizzled receptor
RHOB
CDC42
RHO/ROCK
GTPases
MMP2
Exosome
(containing MMP2)
LRP5/6
W-RAMP
Wnt-mediated receptor
-actin-myosin polarity
structure
Vesicular trafficking
FLNA
Filamin A
Actin binding protein
ROR2
MT1-MMP
Matrix protease

Figure 3

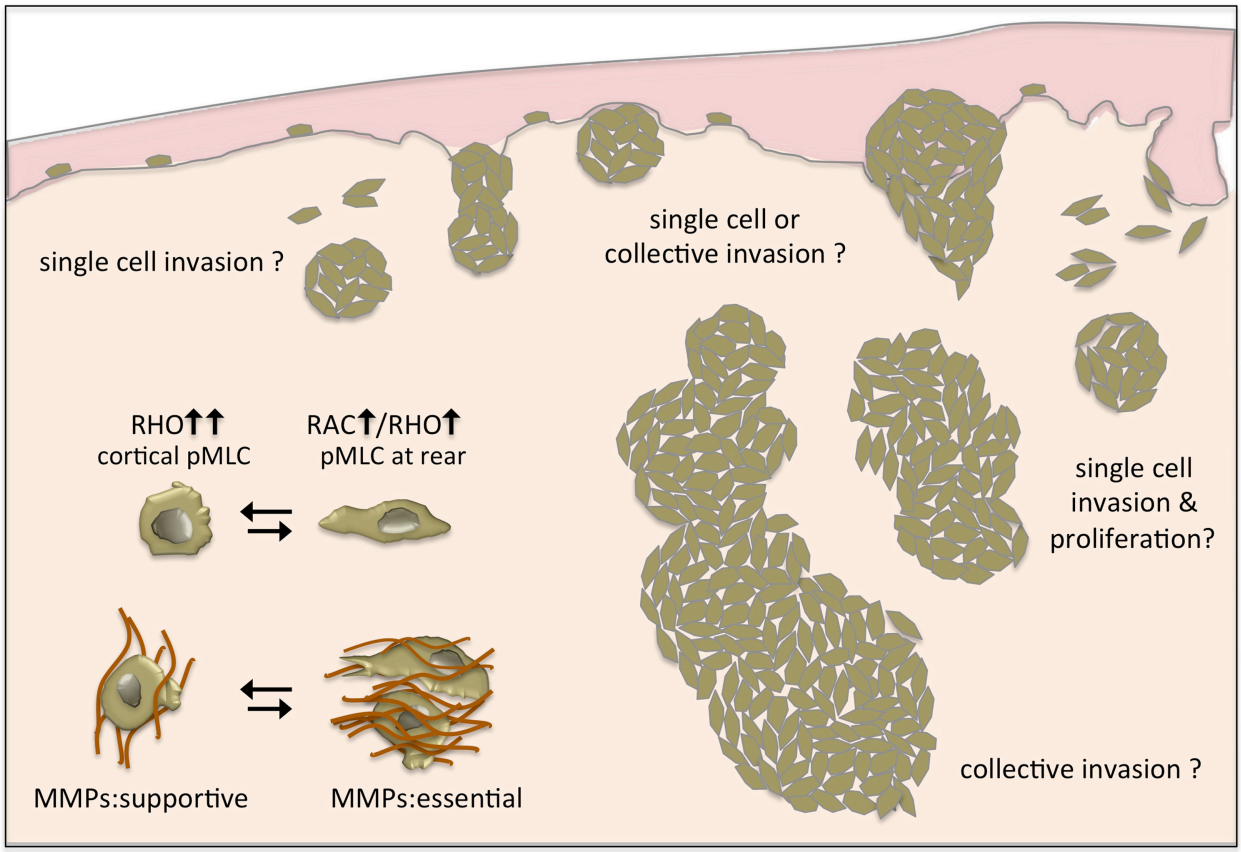
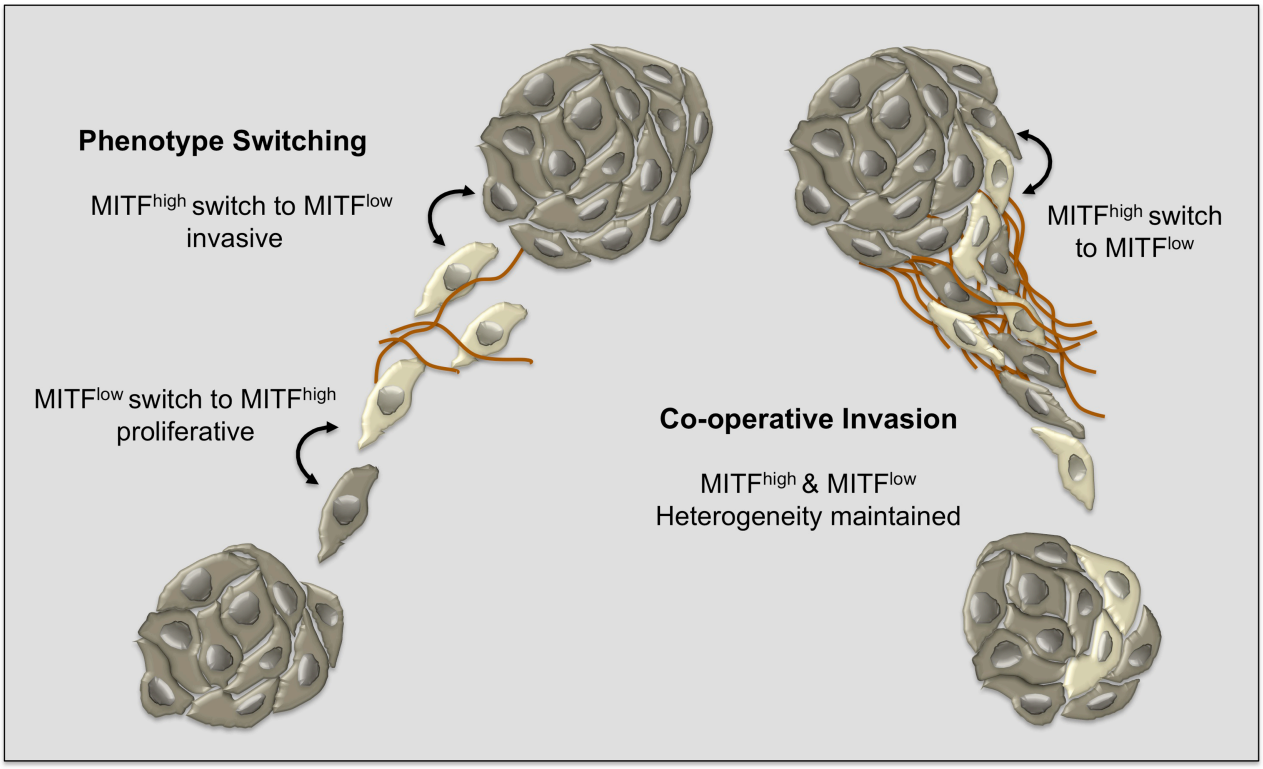

single cell invasion ?
single cell or
collective invasion ?
RHO↑↑
cortical pMLC
RAC↑/RHO↑
pMLC at rear
single cell
invasion &
proliferation?
MMPs:supportive
MMPs:essential
collective invasion ?

Figure 4


Phenotype Switching
MITFhigh switch to MITFlow
invasive
MITFlow switch to MITFhigh
proliferative
MITFhigh switch
to MITFlow
Co-operative Invasion
MITFhigh & MITFlow
Heterogeneity maintained